

# Hypercholesterolemia and changes in lipid and bile acid metabolism in male and female cyp7A1-deficient mice

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**Abstract** Cholesterol 7 $\alpha$ -hydroxylase, a rate-limiting enzyme for bile acid synthesis, has been implicated in genetic susceptibility to atherosclerosis. The gene, *CYP7A1*, encoding a protein with this activity, is expressed normally only in hepatocytes and is highly regulated. Our *cyp7A1* gene knockout mouse colony, as young adults on a chow diet, is hypercholesterolemic. These mice were characterized extensively to understand how *cyp7A1* affects lipid and bile acid homeostasis in different tissue compartments and whether gender plays a modifying role. Both male and female *cyp7A1*-deficient mice had decreased hepatic LDL receptors, unchanged hepatic cholesterol synthesis, increased intestinal cholesterol synthesis and bile acid transporters, and decreased fecal bile acids but increased fecal sterols. In females, *cyp7A1* deficiency also caused changes in hepatic fatty acid metabolism, decreased hepatic canalicular bile acid transporter, Bsep, and gallbladder bile composition altered to a lithogenic profile. Taken together, the data suggest that *cyp7A1* deficiency results in a proatherogenic phenotype in both genders and leads to a pro-lithogenic phenotype in females.—Erickson, S. K., S. R. Lear, S. Deane, S. Dubrac, S. L. Huling, L. Nguyen, J. S. Bollineni, S. Shefer, H. Hyogo, D. E. Cohen, B. Shneider, E. Sehayek, M. Ananthanarayanan, N. Balasubramanian, F. J. Suchy, A. K. Batta, and G. Salen. **Hypercholesterolemia and changes in lipid and bile acid metabolism in male and female *cyp7A1*-deficient mice.** *J. Lipid Res.* 2003. 44: 1001–1009.

**Supplementary key words** liver • intestine • lipid synthesis • low density lipoprotein receptors • sterol 27-hydroxylase • bile acid transporters • fatty acids

Cholesterol 7 $\alpha$ -hydroxylase is a rate-limiting enzyme for both cholesterol catabolism and bile acid synthesis. It has been proposed to play a role in genetic susceptibility to

atherosclerosis based on studies in both humans (1, 2) and experimental animals (3, 4). A gene, *CYP7A1*, encoding a protein with this activity has been cloned from a variety of mammals (5–9), including humans (10, 11). Under normal circumstances, *CYP7A1* is expressed only in the liver (6). It shows a unique developmental pattern of expression (12, 13) and a unique liver lobular distribution in the adult (13, 14). Its regulation is complex [as reviewed in refs. (15, 16)], with much of it occurring at the transcriptional level. The *CYP7A1* gene promoter has been studied extensively, and multiple response sites have been identified [as reviewed in ref. (17)].

A polymorphism in the *CYP7A1* promoter is associated with increased LDL levels in humans (18). Transient overexpression of *CYP7A1* by adenovirus infection in vivo in hamsters (19) and in LDL receptor gene knockout mice (20) resulted in lowered plasma cholesterol, specifically in the LDL fraction; however, stable germ line transmission of *CYP7A1* overexpression in mice led to no net change in plasma cholesterol (21, 22).

Knockout of the *CYP7A1* gene in mice was reported originally to be associated with >90% mortality in the postnatal period, which was prevented by feeding a 2% cholic acid diet (23). The adult *cyp7A1* gene knockout mice were normocholesterolemic (24, 25) with no change in lipoprotein pattern (24) and had a decreased bile acid pool size (24, 25). These mice also exhibited decreased cholesterol absorption, increased in vivo hepatic chole-

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terol synthesis, decreased fecal bile acid, and increased fecal cholesterol excretion (25). Alternative bile acid synthesis did not respond to dietary interventions (26). Cyp7A1 gene knockout mice also were reported to have neonatal cholestasis at postnatal day 5 as a consequence of accumulation of monohydroxy bile acids in their livers (27). Recently, Pullinger et al. (28) described a human CYP7A1 gene truncation resulting in loss of activity. Both heterozygotes and homozygotes for this human mutation were hypercholesterolemic with marked elevation in the LDL fraction.

To gain further insight into the role(s) of *CYP7A1* in the regulation of lipid metabolism, we obtained founder adult mice with the *cyp7A1* gene knockout from Jackson Labs (deposited by David Russell) and set up a breeding colony at the Veterans Affairs Medical Center (VAMC) in San Francisco. The descendants of these mice exhibited much lower mortality with a milder postnatal physical phenotype than described originally despite the maintenance of the colony on a chow diet. Furthermore, in preliminary studies (29–31), we found that these mice had increased plasma cholesterol levels. To determine the origins of this hypercholesterolemia, we asked how *cyp7A1* gene knockout affected lipid and bile acid metabolism in our colony and whether and how gender modified the phenotype.

## METHODS

### Animals

A closed breeding colony was set up at San Francisco VAMC in November, 1997, with three founder mice obtained from Jackson Labs, one male and two females with *cyp7A1* gene knockout on the B6,129S background. Genetic controls were bred in parallel, also as a closed breeding colony, from four B6,129S founder pairs obtained from Jackson Labs at the same time as the knockouts. The *cyp7A1* gene knockout (*cyp7A1*<sup>-/-</sup>) colony was expanded by father-daughter and then brother-sister matings, followed by random matings within the same generation. Overall survival of pups to adulthood to date is >65% (>10 generations). The male-female ratio of survivors within this colony is the same as in the wild-type colony. All mice were maintained on normal rodent chow and water ad lib. Mice were studied at 3–5 months of age. All were killed in the mid-light period. Animals of both genotypes have been studied in parallel since inception of our colonies and at different times throughout the year to minimize environmental effects. All *cyp7A1*<sup>-/-</sup> animals studied to date have been hypercholesterolemic. All protocols were approved by the Animal Studies Subcommittee, VAMC, San Francisco, CA. The Animal Facility at VAMC, San Francisco is AALAC accredited.

### Assays

**Histology.** Liver fat was visualized by Oil Red O stain based on the method of Humason (32). Briefly, a small lobe from livers of age-matched male and female *cyp7A1* gene knockouts and controls was immersion fixed in 10% buffered formalin and frozen in OCT. Frozen sections were cut at 14 microns and floated in water. The free-floating sections were stained for 10 min in a saturated solution of Oil Red O in ethanol. The sections were then rinsed in water and counterstained with Meyer's hematoxylin,

blued with PBS, and mounted on slides for photography. For small intestinal morphology, randomly chosen samples from different areas of the small intestine were fixed, sectioned, and stained with HandE by standard procedures.

**Plasma.** Triglycerides were quantified by enzymatic-endpoint measurements, correcting for free glycerol using Sigma kit Triglyceride (Gpo-Trinder, #337-B). Cholesterol was determined using Sigma kit Infinity cholesterol reagent (#401-500p).

**Gallbladder bile analyses.** Bile salt concentrations and compositions were determined by HPLC utilizing glycocholate as an internal standard (33). Bile salt hydrophobicity index (HI) was determined according to Heuman (34). Biliary cholesterol concentrations were determined using Sigma kit Infinity cholesterol reagent (#401-500p). Concentrations and molecular species of phosphatidylcholines in bile were quantified by electrospray injection mass spectrometry using 15:0-15:0 phosphatidylcholine as an internal standard (35). Cholesterol saturation index (CSI) was calculated according to Carey (36).

**Intestinal bile acid content.** Bile acid contents of individual small intestines and colons were determined by gas liquid chromatography-mass spectrometry (GC-MS) as described previously (37).

**Determination of ileal apical sodium-dependent bile acid transporter and ileal lipid binding protein.** Small intestines were flushed with ice-cold saline immediately after removal. Each was divided into three equal parts: proximal (duodenum/jejunum), middle, and distal (ileum). Ileal protein homogenates and brush border membrane vesicles were prepared from the distal third of mouse intestine (homogenates and brush border membranes also were prepared from the upper and middle thirds for comparison) using previously described methods (38). Brush border membrane enrichment was assessed by measuring alkaline phosphatase in homogenates and membranes. Quantitative Western blot analysis of the apical sodium-dependent bile acid transporter (ASBT) and the ileal lipid binding protein (ILBP) was performed as previously described (39). Antibodies against ASBT and ILBP were generously provided by Drs. Paul Dawson and Michael Crossman, respectively.

**Fecal bile acids and sterols.** Feces were collected individually over 3 days and freeze dried. Sterol and bile acid contents were determined by GC-MS as described previously (37).

**Liver cholesterol and triglycerides.** Liver total, free, and esterified cholesterol and triglycerides were determined following Bligh-Dyer extraction of whole-liver homogenates as described previously (40). Total cholesterol was determined using Sigma kit Infinity cholesterol reagent (#401-500p). Free cholesterol was determined using the Free Cholesterol kit from Wako (#274-47109). Esterified cholesterol was estimated by calculation of the difference between total and free cholesterol. Triglycerides were determined by enzymatic-endpoint measurement using Sigma kit Triglyceride (Gpo-Trinder, #337-B).

**Preparation of liver subcellular fractions.** Microsomes and mitochondria were prepared from flash-frozen liver. Briefly, 0.5–1.0 g liver was homogenized in a Potter-Elyjhem homogenizer with 5 vol of buffer (0.25 M sucrose, 0.1 M Tris, 0.1 mM disodium EDTA, 0.1 mM DTT, pH 7.4). Mitochondria and microsomes were isolated by differential centrifugation (2,000 g to 9,000 g and 10,000 g to 100,000 g, respectively). The pellets were washed and resuspended in storage buffer (0.1 M dipotassium phosphate, 0.05 M KCl, 1 mM DTT, 5 mM disodium EDTA, 20% glycerol, pH 7.4) at 25–30 mg protein/ml.

**LDL receptors.** Receptor-specific LDL binding to liver membranes was determined as described previously (41) using human LDL.

**Enzyme activities.** Cholesterol 7 $\alpha$ -hydroxylase was assayed with consideration of endogenous cholesterol as described previously (42). Briefly, microsomes were incubated with [4-<sup>14</sup>C]cholesterol and an NADPH generating system, followed by hexane extrac-

tion and separation by column and thin-layer chromatography. Mitochondrial cholesterol 27-hydroxylase was assayed using [4-<sup>14</sup>C]cholesterol and a mitochondria-specific NADPH-generating system (isocitrate dehydrogenase) as described previously (42). 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase was assayed using [<sup>14</sup>C]HMG-CoA and an NADPH-generating system followed by separation of the products by thin-layer chromatography as described previously (42).

**Determination of hepatic bile acid transporter mRNA expression.** Ntcp, Bsep, Mrp2, and Lst/Oatp4 mRNA levels were determined by Northern blot essentially as described previously (43) using poly(A)<sup>+</sup> RNA and normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cDNA probes used were: 1) Ntcp: 0.9 kb *Eco*R1 fragment (GenBank M77479) isolated from the full-length cDNA cloned into pBluescript; 2) Bsep: 4.0 kb *Xho*I fragment (GenBank U69487) isolated from the full-length cDNA cloned into pcDNA3; 3) Mrp2: 2.5 kb fragment encoding the C-terminal half of the cDNA (GenBank L49379) amplified by PCR and cloned into pCR2.1.3; 4) Lst-1/Oatp4: a 1.9 kb fragment of the entire open reading frame of the cDNA amplified by PCR (GenBank AF147740) and cloned into pCR2.1; 5) GAPDH: 1.3 kb *Pst*I fragment isolated from a full-length rat cDNA cloned into pBluescript.

**In vivo synthesis of cholesterol and fatty acids.** The <sup>3</sup>H<sub>2</sub>O method was used as described previously (40) except that 40 mCi <sup>3</sup>H<sub>2</sub>O/mouse was used.

**Determination of mRNA levels for LDL receptors *cyp27A1*, *cyp8B*, *CPT-1*, and *AOX-1*.** Total RNA was extracted from flash-frozen livers, equal amounts pooled from five different samples from each gender and genotype, and mRNAs determined by Northern blot as described previously (13) using 1 μg total RNA. The blots were normalized against cyclophilin. The following probes were used: rat *cyp7A1* probes as described previously (13), rat *cyp27* cDNA from N. Avadhani, mouse *cyp8B* cDNA from David Russell, a rat LDL receptor cDNA fragment from John Trawick, rat *AOX-1* cDNA from Deanna Kroetz, and mouse *CPT-1* cDNA from Sonia Najjar.

**Genotyping.** Tail tip DNA was extracted using the Dneasy Tissue Kit from Qiagen (#69504) and analyzed by PCR using primers designed by Jackson Labs (IMR0013, IMR0014, IMR0594, and IMR0595) and the Advantage cDNA PCR kit from Clontech (#K1905-1). P cycling conditions were those as suggested by Jackson Labs.

**Statistics.** Paired comparisons of means were done using Student's *t*-test. Multiple comparisons were made by ANOVA.

## RESULTS

### Effects on liver morphology

No differences were noted in body weight between adult homozygote *cyp7A1* gene knockout and wild-type mice (Table 1), although as expected, female body weights of both genotypes were lower than those of males ( $P < 0.0001$ ). Mild liver hypertrophy was found consistently in both male and female knockout mice, ~24% increase when corrected for body weight (Table 1). Male knockout and wild-type mouse whole-liver free or esterified cholesterol and triglyceride levels were similar, but cholesterol esters were modestly increased in female knockout mouse livers, with a corresponding decrease in free cholesterol (Table 1). The lobular distribution of lipids determined by Oil Red O stain consistently showed larger droplets in the knockout livers of both sexes (Fig. 1).

### Effects on plasma lipids

Male knockout mice had ~34% increase in plasma cholesterol levels,  $P < 0.001$ , and female knockout mice, ~50%,  $P < 0.001$ , (Table 1). Plasma triglycerides were similar in male knockout mice and wild-type mice, but they were decreased in knockout females ~43%,  $P < 0.005$ , relative to wild-type mice (Table 1).

### Effects on fecal bile acids and sterols

Amounts of food eaten/day/g body weight and amounts of feces/day/g body weight were similar in wild-type and knockout mice (data not shown). The male knockout mice fecal bile acid pools averaged ~54% of wild-type mice,  $P < 0.002$ , while the female knockout mice pools were ~28% of wild-type mice,  $P < 0.002$  (Table 2). Fecal bile acid composition profiles were not remarkably different between wild-type and knockout mice of either sex, but amounts of the different bile acids generally were decreased in knockout mice (Fig. 2). Male and female knockout mice had ~3-fold increase in fecal cholesterol,  $P < 0.001$ , and ~2-fold, in coprostanol, a gut metabolite of cholesterol,  $P < 0.01$  (Table 2). Little difference was ob-

TABLE 1. Effects of *Cyp7A1* gene knockout and gender on body and liver weights, plasma and hepatic cholesterol and triglycerides

	Body Weight	Liver Weight	Liver Free Cholesterol	Liver Cholesterol Ester	Liver Triglyceride	Plasma Cholesterol	Plasma Triglyceride
	g	% body weight		μg/mg protein		mg/dl	
Male wild-type mice	30.8 ± 0.5 (n = 37)	4.00 ± 0.05 (n = 36)	9.0 ± 0.2 (n = 5)	1.4 ± 0.4 (n = 5)	45.1 ± 5.6 (n = 5)	94 ± 6 (n = 26)	76 ± 5 (n = 26)
Male knockout mice	29.3 ± 0.8 (n = 35)	5.03 ± 0.08 <sup>a</sup> (n = 34)	9.8 ± 0.6 (n = 5)	1.8 ± 0.6 (n = 5)	46.7 ± 4.4 (n = 5)	126 ± 8 <sup>b</sup> (n = 27)	61 ± 4 (n = 27)
Female wild-type mice	23.0 ± 0.4 (n = 29)	3.82 ± 0.07 (n = 29)	12.5 ± 0.5 (n = 5)	1.5 ± 0.4 (n = 5)	61.8 ± 6.4 (n = 5)	86 ± 5 (n = 18)	42 ± 4 (n = 18)
Female knockout mice	24.6 ± 0.9 (n = 37)	4.67 ± 0.09 <sup>a</sup> (n = 37)	9.9 ± 0.6 <sup>b</sup> (n = 6)	3.5 ± 0.6 <sup>a,b</sup> (n = 5)	69.0 ± 4.2 (n = 5)	132 ± 5 <sup>a</sup> (n = 32)	24 ± 4 <sup>c</sup> (n = 32)

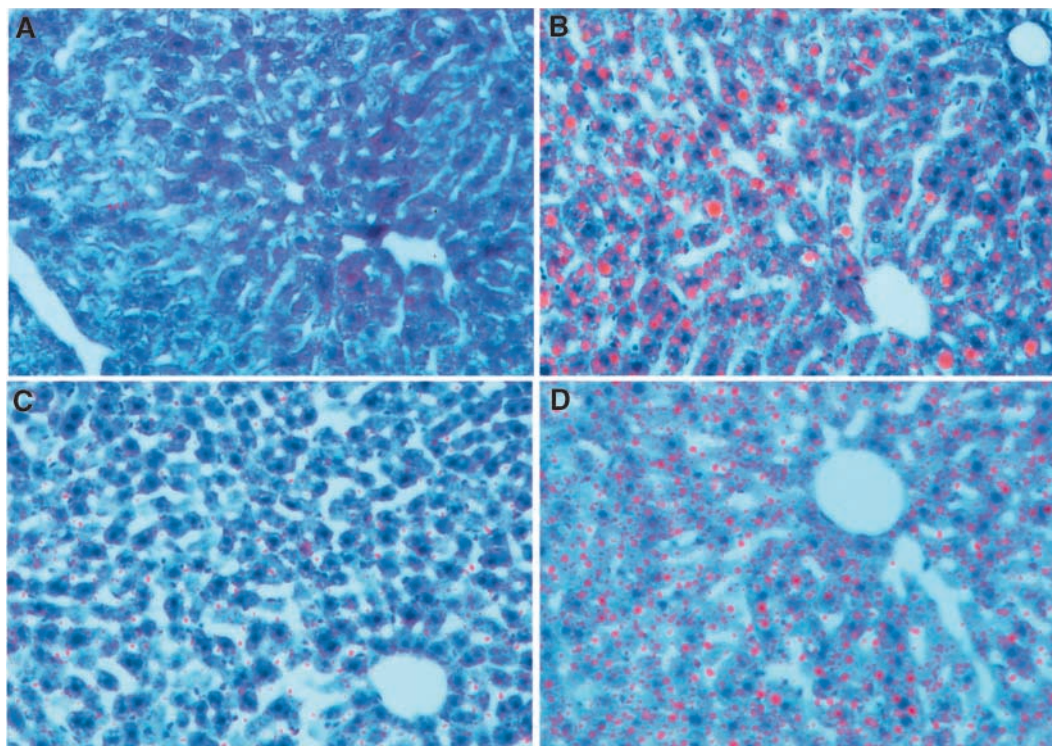
Numbers in parentheses are the number of animals.

<sup>a</sup>  $P < 0.0001$ , wild-type mice versus knockout mice.

<sup>b</sup>  $P < 0.001$ , wild-type mice versus knockout mice.

<sup>c</sup>  $P < 0.005$ , wild-type mice versus knockout mice.





**Fig. 1.** Changes in fat distribution in livers of *cyp7A1* gene knockout male and female mice on a chow diet as determined by Oil Red O stain. Small lobes of livers from male and female wild-type ( $+/+$ ) and *cyp7A1* gene knockout ( $-/-$ ) mice were fixed and stained as described in Methods. A: Wild-type male mice. B: Knockout male mice. C: Wild-type female mice. D: Knockout female mice. Representative sections are shown.

served in fecal campesterol, stigmasterol, sitosterol, or sitostanol concentrations.

#### Effects on gallbladder bile composition

Composition of gallbladder bile from wild-type mice was gender dependent. Bile salts (taurine conjugates) were increased  $\sim 1.3$ -fold,  $P < 0.01$ , cholesterol,  $\sim 1.8$ -fold,  $P < 0.0001$ , and phosphatidyl choline,  $\sim 1.6$ -fold,  $P < 0.0001$  in females relative to males. The net result was little gender difference in CSI (Table 3). Gender was an important modifier in knockout mice. Gallbladder bile composition in male knockout mice relative to wild-type mice was little changed; however, gallbladder bile from knockout females relative to that from wild-type mice had a 44% decrease in bile salts,  $P < 0.001$ , and a 62% decrease in phosphatidyl choline,  $P < 0.001$ , with little change in cholesterol concentration. The net result was a 2.5-fold increase in CSI in

the knockout female mice,  $P < 0.01$ , and a 2.1-fold increase relative to male knockout mice,  $P < 0.01$ .

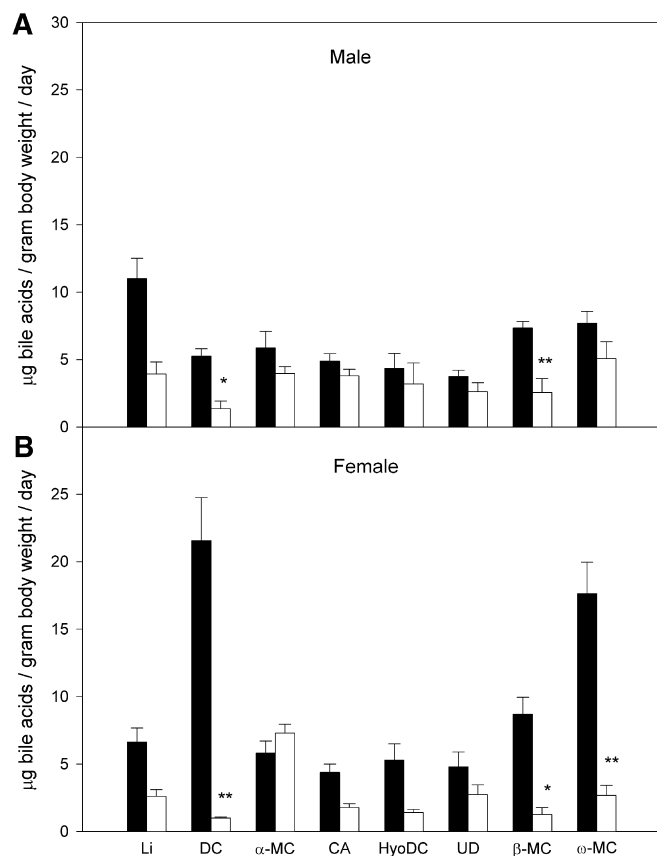
Bile acid composition in gallbladder bile from female knockout mice relative to female wild-type mice showed no change in amounts of cholate or muricholate, but  $\sim 50\%$  decrease in ursodeoxycholate,  $P < 0.01$ ;  $\sim 85\%$  decrease in chenodeoxycholate,  $P < 0.05$ ; and  $\sim 80\%$  decrease in deoxycholate,  $P < 0.01$ . In male wild-type mice, gallbladder bile had a muricholate-cholate ratio of  $\sim 1.8$  (compared with  $\sim 1.0$  in female wild-type and knockout mice), while the ratio in male knockout mouse bile was increased to  $\sim 3.9$ . The only other change in male knockout mouse bile acid composition was  $\sim 60\%$  decrease in deoxycholate relative to wild-type mice,  $P < 0.05$ . The net result in male knockout mice was a decreased HI, while that in the female knockout mice was unchanged (Table 3). The molecular species of phosphatidyl choline present in gall-

TABLE 2. Effects of *Cyp7A1* gene knockout and gender on fecal bile acid and fecal sterol excretion

	Total Bile Acids	Total Sterols	Cholesterol	Coprostanol
	$\mu\text{g/day/g body weight}$			
Male wild-type mice (n = 10)	50 $\pm$ 3	341 $\pm$ 18	59 $\pm$ 4	75 $\pm$ 4
Male knockout mice (n = 3)	27 $\pm$ 3 <sup>a</sup>	568 $\pm$ 46 <sup>b</sup>	193 $\pm$ 36 <sup>b</sup>	140 $\pm$ 2 <sup>b</sup>
Female wild-type mice (n = 10)	75 $\pm$ 8	288 $\pm$ 22	40 $\pm$ 3	64 $\pm$ 5
Female knockout mice (n = 5)	21 $\pm$ 1 <sup>b</sup>	392 $\pm$ 18 <sup>b</sup>	148 $\pm$ 15 <sup>b</sup>	94 $\pm$ 5 <sup>b</sup>

<sup>a</sup>  $P < 0.002$ , wild-type mice versus knockout mice.

<sup>b</sup>  $P < 0.001$ , wild-type mice versus knockout mice.



**Fig. 2.** Fecal bile acid composition in *cyp7A1*-deficient mice. Feces were collected and analyzed individually for bile acid composition by gas liquid chromatography-mass spectrometry as described in Methods. A: wild-type male mice, solid bars,  $n = 10$  and knockout males, open bars,  $n = 3$ . B: Wild-type females, solid bars,  $n = 10$  and knockout females, open bars,  $n = 5$ . Li, lithocholic; DC, deoxycholic;  $\alpha$ -MC,  $\alpha$ -muricholic; CA, cholic; HyoDC, hyodeoxycholic; UD, ursodeoxycholic;  $\beta$ -MC,  $\beta$ -muricholic;  $\omega$ -MC,  $\omega$ -muricholic. \*  $P < 0.01$ ; \*\*  $P < 0.001$ .

bladder bile showed neither gender- nor genotype-dependent changes.

### Effects on intestinal metabolism

No changes were found in small intestinal morphology (data not shown), and small intestine and colon weights in knockout mice were similar to wild-type mice. Intestinal cholesterol synthesis measured *in vivo* was increased in both knockout male mice,  $3.4 \pm 0.3 \mu\text{mol } ^3\text{H}_2\text{O/g/h}$ ,  $n = 9$ ,

versus  $1.4 \pm 0.7$ ,  $n = 10$ ,  $P < 0.03$ , for wild-type mice, and knockout female mice,  $2.1 \pm 0.1 \mu\text{mol } ^3\text{H}_2\text{O/g/h}$ ,  $n = 10$ , versus  $1.5 \pm 0.1$ ,  $n = 10$ ,  $P < 0.01$ , for wild-type mice. Intestinal fatty acid synthesis was little affected.

Similar amounts of bile acids were recovered in small intestine of both knockout and wild-type mice irrespective of gender:  $1.9 \pm 0.2 \mu\text{g}$  bile acids/g tissue ( $n = 20$ ) versus  $1.3 \pm 0.8$  ( $n = 4$ ). Fewer bile acids were recovered in the knockout mouse colons:  $0.6 \pm 0.1 \mu\text{g}$  bile acids/g tissue ( $n = 20$ ) versus  $1.0 \pm 0.1$  ( $n = 4$ ),  $P < 0.01$ , for wild-type mice.

Ileal brush border membrane vesicle preparation was similar in male and female mice, whether wild-type or knockout. ASBT protein was increased 2.8-fold in male knockout mice,  $P < 0.01$ , and 2.6-fold in female knockout mice,  $P < 0.001$  (Fig. 3), with no significant gender difference in either genotype. ILBP protein was decreased 54% in male knockout mice,  $P < 0.01$ , and 30% in female knockout mice,  $P < 0.01$  (Fig. 3). Although no statistically significant gender differences were found in wild-type mice, ILBP in knockout mouse females was  $\sim 2$ -fold higher than in knockout mouse males,  $P < 0.03$  (Fig. 3).

### Effects on hepatic metabolism

mRNA levels for Bsep, the major hepatic bile acid canalicular transporter, were higher in female wild-type mice than in male mice:  $0.20 \pm 0.01$  units,  $n = 5$ , versus  $0.09 \pm 0.02$  units,  $n = 5$ ,  $P < 0.001$ . They were decreased  $\sim 30\%$ ,  $P < 0.002$ , in female knockout mice, but unchanged in male knockout mice. The canalicular transporter, Mrp2, was unaffected as were the basolateral transporters Ntcp and Lst-1/Oatp4.

Liver cholesterol synthesis measured *in vivo* was similar in wild-type and knockout mice of either gender as was activity of HMG-CoA reductase, a rate-limiting enzyme for cholesterol synthesis (Table 4).

Cholesterol  $7\alpha$ -hydroxylating activity was detectable in knockout livers, albeit at  $\sim 9\%$  (females) and  $\sim 16\%$  (males) of wild-type liver levels (Table 4). The *cyp7A1* knockout mice used in this study were homozygous for the mutated inactive *cyp7A1* gene, as shown by genotyping, and lacked detectable *cyp7A1* in liver mRNA by Northern blot (data not shown).

Cholesterol 27-hydroxylase activity was increased  $\sim 90\%$  in knockout mouse males,  $P < 0.01$ , and  $\sim 57\%$  in knockout mouse females,  $P < 0.001$  (Table 4). *Cyp27A1* mRNA level and gender distribution were unchanged.

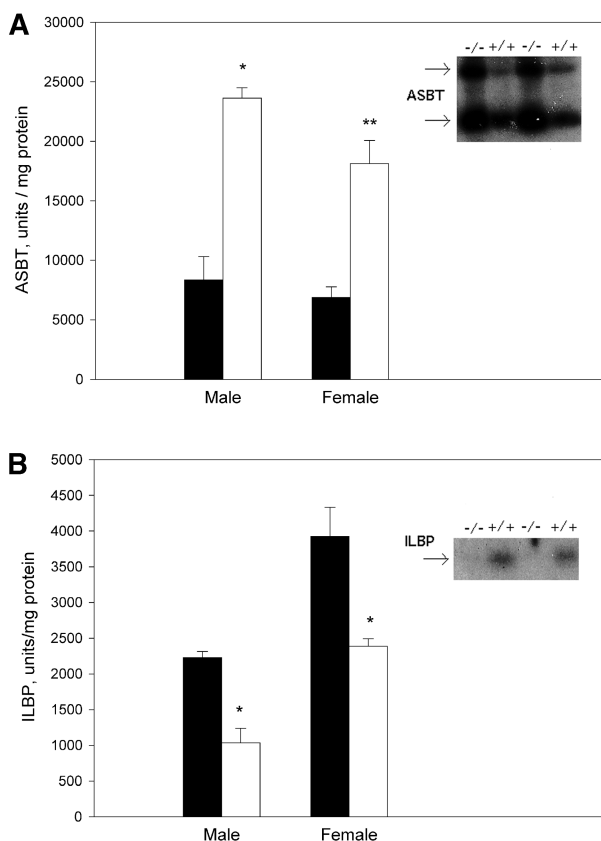
TABLE 3. Effects of *Cyp7A1* gene knockout and gender on gallbladder bile composition

	Bile Salts	Cholesterol	Phosphatidylcholine	CSI	HI
	<i>mM</i>				
Male wild-type mice ( $n = 5$ )	$54.4 \pm 4.2$	$2.4 \pm 0.3$	$9.2 \pm 0.8$	$87 \pm 10$	$-0.47 \pm 0.01$
Male knockout mice ( $n = 6$ )	$49.0 \pm 2.6$	$2.8 \pm 0.4$	$9.6 \pm 2.1$	$116 \pm 29$	$-0.61 \pm 0.01^a$
Female wild-type mice ( $n = 6$ )	$71.4 \pm 3.2$	$4.3 \pm 0.2$	$14.5 \pm 0.7$	$97 \pm 6$	$-0.37 \pm 0.02$
Female knockout mice ( $n = 5$ )	$39.8 \pm 5.4^a$	$3.1 \pm 0.6$	$5.5 \pm 1.9^a$	$245 \pm 59^b$	$-0.36 \pm 0.02$

CSI, cholesterol saturation index; HI, hydrophobicity index of bile salt content. Numbers in parentheses are the number of animals.

<sup>a</sup>  $P < 0.001$ , wild-type mice versus knockout mice.

<sup>b</sup>  $P < 0.01$ , wild-type mice versus knockout mice.



**Fig. 3.** Effects of *cyp7A1* deficiency on intestinal bile acid transport systems. Ileal brush border membranes were prepared and analyzed for apical sodium-dependent bile acid transporter (ASBT) and ileal lipid binding protein (ILBP) by Western blot as described in Methods.  $n = 5$  for each gender and genotype. A: ASBT. B: ILBP. Insets: representative Western blots for each protein. Wild-type mice, solid bars; knockout mice, open bars. \*  $P < 0.01$ ; \*\*  $P < 0.001$ .

Cyp8B, encoding sterol 12 $\alpha$ -hydroxylase, which determines the amount of cholic acid synthesized, was more highly expressed in knockout mouse livers; relative mRNA levels were 3.1 units versus 1.0 for males and 5.0 units versus 1.0 for females.

Liver fatty acid synthesis was increased  $\sim 2$ -fold in female knockout mice,  $P < 0.01$ , with no change in male (Table 4). Fatty acid oxidation indices, mRNA levels for CPT-1 (encoding carnitine palmitoyl transferase-1, a reflection of mitochondrial  $\beta$ -fatty acid oxidation capacity) and

AOX-1 (encoding acyl CoA oxidase, a reflection of peroxisomal  $\beta$ -fatty acid oxidation capacity) were increased in female knockout mice, but not in male (Table 4).

Hepatic LDL receptors, measured using an  $^{125}\text{I}$ -LDL-specific binding assay, were decreased  $\sim 80\%$  in male knockout mice,  $P < 0.001$ , and  $\sim 40\%$  in female knockout mice,  $P < 0.02$  (Fig. 4). LDL receptor mRNA levels also were decreased,  $\sim 70\%$  in male knockout mice and  $\sim 30\%$  in female knockout mice (Fig. 4). Changes attributable to gender did not reach statistical significance in either wild-type or knockout mice as determined by specific binding, although mRNA levels were higher in livers from both wild-type and knockout females compared with males (Fig. 4).

## DISCUSSION

In contrast to results reported for the original *cyp7A1* gene knockout mouse colony (23–26), ours was hypercholesterolemic. Differences between the phenotypes of these two colonies likely reflect as yet undefined differences in their genetic backgrounds or in their environment. The influence of genetic background on phenotype is well known; of particular relevance to this work are the studies of Dueland et al. (3) and Machleder et al. (4). At least one report of phenotypic differences in genetically identical mice has been attributed to change in environment (44).

The original description of these mice (23) reported  $\sim 90$ – $95\%$  postnatal mortality;  $\sim 5$ – $10\%$  of the mice that were born survived without vitamin or cholate supplementation to the mothers. This suggested a “leaky” phenotype. At least one other example of variable survival has been reported for gene knockout of a lipid-related gene, that for SREBP-1 (45). We chose to maintain our colony on a chow diet to select for the phenotype of the 5–10% of pups originally reported to survive when the mothers were maintained on chow (23). The female founders of our colony and all their adult descendants have been hypercholesterolemic. This suggests that this colony is a good model for human CYP7A1 deficiency, which also exhibits a hypercholesterolemic phenotype (28).

Hypercholesterolemia in these mice is explained, at least partially, by the decreased numbers of hepatic LDL receptors. This mirrors the result in mouse *cyp7A1* over-

TABLE 4. Effects of *Cyp7A1* gene knockout and gender on hepatic cholesterol metabolism

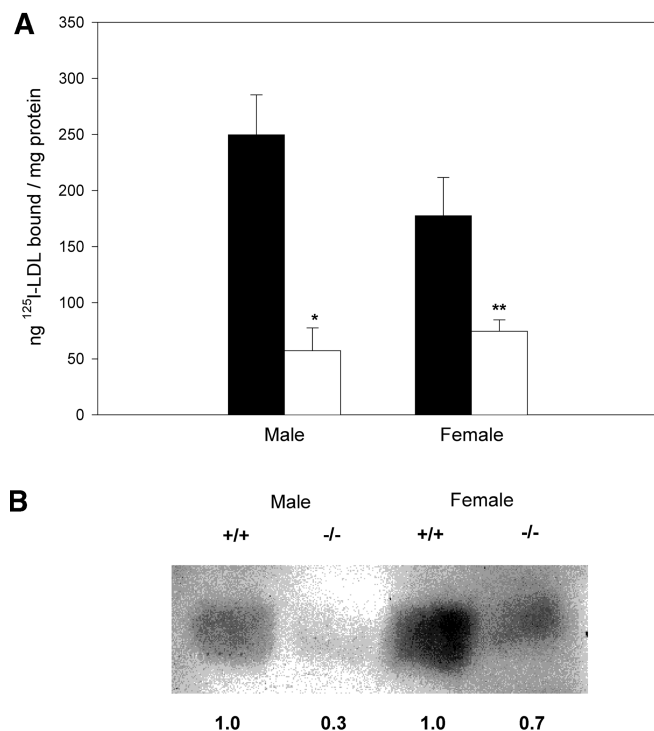
Animals	HMG CoA Reductase	Cholesterol 7 $\alpha$ -Hydroxylase	Cholesterol 27-Hydroxylase	Cholesterol Synthesis	Fatty Acid Synthesis	Fatty Acid Oxidation Index	
						CPT-1	AOX1
		<i>pmol/min/mg protein</i>		<i><math>\mu\text{mol/g liver/h}</math></i>		<i>mRNA, units</i>	
Male wild-type mice	117 $\pm$ 28 (n = 7)	21.5 $\pm$ 2.4 (n = 7)	19.0 $\pm$ 3.2 (n = 3)	5.3 $\pm$ 0.8 (n = 10)	52.5 $\pm$ 11.0 (n = 10)	1.0	1.0
Male knockout mice	93 $\pm$ 9 (n = 10)	3.4 $\pm$ 0.2 <sup>a</sup> (n = 10)	36.6 $\pm$ 4.3 <sup>b</sup> (n = 9)	5.4 $\pm$ 0.3 (n = 9)	57.8 $\pm$ 6.0 (n = 9)	1.1	0.8
Female wild-type mice	115 $\pm$ 23 (n = 6)	29.5 $\pm$ 2.2 (n = 6)	22.2 $\pm$ 2.1 (n = 3)	5.8 $\pm$ 1.0 (n = 10)	26.7 $\pm$ 4.7 (n = 10)	1.0	1.0
Female knockout mice	90 $\pm$ 22 (n = 8)	2.7 $\pm$ 0.1 <sup>a</sup> (n = 8)	34.9 $\pm$ 1.5 <sup>a</sup> (n = 8)	4.0 $\pm$ 0.4 (n = 10)	51.8 $\pm$ 6.5 <sup>b</sup> (n = 5)	2.6	1.6

Numbers in parentheses are the number of animals.

<sup>a</sup>  $P < 0.001$ , wild-type mice versus knockout mice.

<sup>b</sup>  $P < 0.01$ , wild-type mice versus knockout mice.





**Fig. 4.** Hepatic LDL receptor number and mRNA level in *cyp7A1*-deficient mice. Membranes were prepared and analyzed for LDL receptor number using <sup>125</sup>I-LDL-specific binding as described in Methods. Total RNA was extracted, and equal amounts pooled from five livers and analyzed by Northern blot as described in Methods. Blots were normalized to cyclophilin. A: LDL receptor binding; *n* = 6 in each group. Wild-type mice, solid bars; knockout mice, open bars. Male knockout versus male wild-type mice, \* *P* < 0.001; female knockout versus female wild-type mice, \*\* *P* < 0.02. B: LDL receptor mRNA levels. The fold change relative to wild-type mice = 1.0 is noted under each lane.

expressers (21, 22), in which hepatic LDL receptors were increased. These findings, taken together, suggest that hepatic LDL receptor regulation is closely linked to *cyp7A1*.

Like the human CYP7A1-deficient family (28), these mice showed little change in HMG-CoA reductase activity, a rate-limiting enzyme for cholesterol synthesis. In the mice, this was shown to be physiologically relevant by the minimal effects on *in vivo* hepatic cholesterol synthesis. This suggests that hepatic cholesterol synthesis and LDL receptors can be regulated independently. Indeed, in mouse liver, the level of LDL receptors is not obligatorily linked to either a low or a high rate of cholesterol synthesis; LDL receptor-deficient mice show little change in hepatic cholesterol synthesis *in vivo* (46). Taken together, these findings suggest a rationale for the statin resistance observed in homozygote CYP7A1-deficient humans (28): if LDL receptors in these livers are set at a low level, as they are in the *cyp7A1*-deficient mice, and their regulation is uncoupled from the rate of cholesterol synthesis, then inhibiting HMG-CoA reductase/cholesterol synthesis with statins will not result in compensatory up-regulation of hepatic LDL receptors and lowered plasma LDL.

As was found in the original *cyp7A1* gene knockout

mouse colony (24–26), fecal bile acids were decreased, suggesting that at steady state, *de novo* bile acid synthesis rate is lower, as would be expected for loss of a rate-limiting enzyme for bile acid synthesis; however, an additional explanation for decreased fecal bile acids is that intestinal uptake was more efficient. The finding of lower colonic bile acid content coupled with markedly decreased amounts of secondary bile acids in gallbladder bile (suggesting that a high proportion of primary bile acids was removed before being acted upon by intestinal bacteria) supports this. Indeed, the ASBT was increased ~2-fold. This, together with bile acid *de novo* synthesis via the *cyp27A1* and second cholesterol 7 $\alpha$ -hydroxylating pathway(s), appeared to have been sufficient for maintenance of levels of bile acids consistent with survival under normal physiological conditions, including reproduction.

Increased ASBT is consistent with negative feedback regulation of this transporter by bile acids (47, 48) while ILBP is typically under their positive feedback control (49, 50), suggesting that changes in both these proteins reflect a decreased bile acid regulatory pool. On the basis of previously published work (39), it is unlikely that the changes in ASBT and ILBP reflect simple changes in luminal bile salt content. *Cyp7A1* deficiency during development may have affected basal levels of these proteins; in the rat, ASBT (38) and *cyp7A1* (13) have similar developmental patterns of expression in the late fetal/early postnatal stages, suggesting they are linked.

Higher Bsep mRNA level in female wild-type mouse livers compared with male wild-type mouse has not been reported previously and suggests that the increased bile salt concentration in female wild-type mouse gallbladder bile relative to that in male is due to increases in this major canalicular bile salt transporter. These gender differences were no longer evident in knockout mice and are in part responsible for the increased CSI in female knockout mouse gallbladder bile, an index of increased lithogenicity.

Cholesterol 27-hydroxylation is the first step in the alternative or acidic bile acid synthetic pathway; however, sterol 27-hydroxylase (*cyp27A1*) also has an important role in side-chain cleavage [as reviewed in ref. (16)]. Cholesterol 27-hydroxylase activity was increased by *cyp7A1* deficiency in these mice, in contrast to what was reported for the Dallas colony (26), with no increase in *cyp27A1* mRNA level. This suggests that the increased activity is due to posttranslational modification(s) and/or substrate availability. This increase may be responsible in part for some of the differences in phenotype between the two colonies.

Of importance, this colony, like the human family with CYP7A1 deficiency (28), did have hepatic cholesterol 7 $\alpha$ -hydroxylating activity. The identity of the protein(s) responsible is unknown; however, it is likely that other CYP genes encode proteins capable of 7 $\alpha$ -hydroxylating cholesterol.

Studies of *cyp8B* gene knockout mice demonstrated that cholic acid plays an important role in the regulation of bile acid synthesis in this species (51). The increased *cyp8B*

mRNA level in our cyp7A1-deficient mice supports this. These levels did not correlate with amounts of cholic acid in either gallbladder bile or feces, suggesting that a 3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -trihydroxy steroid also may be important. 5 $\beta$ -Cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol was shown recently to be a high-affinity endogenous ligand for pregnane X receptor (52).

Gender differences in fatty acid metabolism are well documented (53). Although both male and female knockout mouse livers had morphological changes in lipid pattern (Fig. 1), hepatic fatty acid metabolism was altered only in the females. Their increased fatty acid synthesis, coupled with increased mRNA levels for two fatty acid oxidation index genes, CPT-1 and AOX-1, suggests that cyp7A1 deficiency in female livers leads to dysregulation in the coordination of fatty acid synthesis and oxidation.

In summary, lack of a functional cyp7A1 gene product had major effects in a variety of tissue compartments including the plasma, liver, gallbladder, and intestine. These changes reflect strategies the organism has utilized to maintain homeostasis and to ensure viability and reproductive capacity. This has been done at the expense of introducing a proatherogenic phenotype in young adult mice of both genders on a chow diet and a prolithogenic phenotype in the females. In addition, cyp7A1 deficiency resulted in a mildly hypertrophied, mildly fatty liver and, in the females, altered fatty acid metabolism. Thus, cyp7A1 deficiency likely results in genetic vulnerability to development of atherosclerosis, cholesterol gallstone disease, and fatty liver disease. In the broader context, these mice, together with those of the Dallas colony (see 25, 26) will provide important information on how the regulation of cholesterol, bile acid, and fat metabolism is integrated to maintain homeostasis at optimal levels. ■

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